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EXTERNAL FACTORS, PRODUCED BY GROWING NERVES TRIGGER A REGENERATIVE RESPONSE IN A NON-REGENERATIVE CENTRAL NERVOUS SYSTEM: PURIFICATION AND MODE OF ACTION.

ANNUAL REPORT

MICHAL SCHWARTZ, Ph.D

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Dept of Neurobiology,
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Mammalian central nervous system (CNS) neurons have negligible posttraumatic regenerative capacity while the nerves of lower vertebrates, as well as the peripheral nervous system (PNS) of mammals regenerate spontaneously after the injury.We found that application soluble substances derived from growing nerves,(in the form of conditioned media, of regenerating fish optic nerves and of newborn rabbit optic nerves to injured adult rabbit optic nerves.The regeneration-like response involved a general increase in protein synthesis in the retina;selective increase in synthesis of a few polypeptides in the retina;and sprouting from the retina in vitro.We succeeded to demonstrate during the first year of this support that the active conditioned media causes environmental modifications manifested by: (a) the appearance of new fibers adjacent to astrocytic processes in area which otherwise seemed to be a scar tissue area (b) change in the profile of the nerve-derived proteins, e.g. an increase in a 12 KDa polypeptide which might be a second mediator in the cascade of events leading to regeneration, (c) increase in thymicine incorporation (d) increase in laminin distribution- a key extracellular matrix protein required for growth.

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FOREWORD

In conducting the research described in this report, the investigators adhere to the "Guide for the care and use of laboratory animals", prepared by the committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW publication No. (NIH)78-23, Revised 1978).

ABBREVIATIONS.

CNS-Central nervous system

PNS-Peripheral nervous system

FCS-Fetal calf serum

NGF-Nerve growth factor

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SUMMARY

Mammalian central nervous system (CNS) neurons have negligible post-traumatic regenerative capacity while the nerves of lower vertebrates, as well as the peripheral nervous system (PNS) of mammals regenerate spontaneoulsy after injury.

Studies in our laboratory as well as in other laboratories carried out in both CNS and PNS provide strong evidence that the microenvironment of the injured nerves (including the cellular elements, soluble substances and the extracellular matrix) have an important role in determining the ability of neurons to regenerate after axonal injury.

In the two years which preceeded the present work we were able to demonstrate that introduction of a presumably supportive environment derived from a regenerating nerve, to injured mammalian CNS induces in the later a regeneration-like response. Specifically we applied soluble substances derived from growing nerves,(in the form of conditioned media, of regenerating fish optic nerves and of newborn rabbit optic nerves) to injured adult rabbit optic nerves. Application was accomplished by the use of a silicone tube (in a form of a wrap around implant) coated inside with collagen and soaked with conditioned media. The regeneration-like response involved a general increase in protein synthesis in the retina; selective increase in synthesis of a few polypeptides in the retina; and sprouting from the retina in vitro.

With this observation we proposed that it might be possible to circumvent the inability of mammalian CNS neuron to regenerate by application of exogenous factors derived from growing nerves. We therefore carried out during the first year of this support studies aiming at finding out the extent of regeneration induced by the soluble factor, establishing the factors mode of action and their identity.

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1. Statement of the problem

1.1 The general problem and the significance of its research

The nerves of the mammalian central nervous system very rarely repair themselves after injuries which comprise a considerable proportion of combat casualties and are the most common cause of severe permanent post-traumatic neuromotor paralysis. These injuries, which include brain, spinal cord and major sense organ trauma, cause permanent disability because the adult mammalian central nervous system is incapable of functional regeneration. Due to the poor regenerative capacity of mammalian CNS, an injury in the lower spinal cord often results in the permanent confinment of the casualty to a wheel chair. Laceration in the upper spinal cord leads to paralysis of the four limbs and a cut in the optic nerve leads to complete incurable blindness. Furthermore, the inability of the central nervous system to regenerate is the main reason why neural transplantation operations can't be performed.

Means which will induce central nervous system regeneration will be of immense benefit to soldiers suffering from these common combat injuries of the head, spinal cord and major sense organs. Success in research of CNS injuries will be a major step in the direction of medical intervention to achieve central nervous system regeneration.

1.2 The basic research problem

The process of axonal regeneration involves (i) establishment of a neuronal growth state and formation of new sprouts, (ii) elongation of new sprouts, and (iii) target recognition, synaptogenesis and cessation of axonal elongation. Successful regeneration probably depends on a synchronized progression of the events that are involved in these major phases. Malfunction of any of these events or lack of synchrony may hamper the process of regeneration.

Some of the events involved in nerve regeneration are reminiscent of development. The cascade of events which leads to growth and elongation may be triggered by one signal, or each of the sequential events may be triggered by a distinct signal. Signals may be provided either by the axonal injury itself or by injury-stimulated changes in the environment.

Our studies as well as studies carried out in various laboratories provide evidence that injury stimulated changes in th environment have a key role in regeneration. Studies on regeneration have, therefore been focused on identifying the elements which are involved in the glial response to injury (cellular, soluble and extracellular non-soluble). The information accumulated allows us to visualize various ways of intervention after CNS injury (1) supply of neurotrophic factors which may save neurons from secondary cell death or which activate neurons to grow (2) provide glial cells with growth factors which may modulate their response to the injury and thereby allow them to acquire growth supportive features, such as production of laminin or other substances needed for growth.

Purification of the factors, studies of their mode of action and regulation should eventually permit convergent multiple interventions to repair damage CNS tissue.

2. Background

Recent studies carried out in various laboratories including our laboratory provide evidence that the environment which surround the injured nerve has a significant influence on determining the regenerating capacity of the nerve.

The emphasis in study of regeneration of CNS neurons is therefore focused on identifying the factors which allow the neurons to regenerate

and on attempting to devise ways to circumvent the impediments to central nerves regrowth.

2.1 Environmental elements and regeneration.

As a result of an injury, the neuron is deprived from target-derived substances and the mutual relationship with the environment is disrupted. The state of growth of the surrounding non-neuronal cells is altered. (Nathaniel & Nathaniel, 1973; Nathaniel & Nathaniel, 1981; Nathaniel & Pease, 1963; Neumann et al., 1983a; Stevenson & Yoon, 1978). This may lead to formation of an environment hostile to regeneration due to the appearance of either a scar tissue (composed of collagen or glia) or axonal growth inhibitors (McConnell & Berry, 1982). Alternatively, the glia proliferation may lead to formation of a growth-supportive environment (Manthorpe et al., 1983; Schwartz et al., 1985; Varon et al., 1983; Williams et al., 1983). It appears that the formation of both the hostile and the supportive environments may occur at different time periods after injury (Neumann et al., 1983a). It is the net outcome of these opposing contributions which may have an impact on the nature of the response to the injury, i.e, regeneration or degeneration.

Surgical manipulations were performed to determine whether a dense glial scar which is formed by the proliferating glial cells interferes with outgrowth of neurites in regeneration. These studies showed that such a scar does not represent a major obstacle to axonal growth (Reier, 1979; Reier et al., 1983). However, there is no conclusive information as to the contribution of scarring to the failure of regeneration in mammalian CNS (Billingsley & Mandel 1982; Krikorian et al.,, 1981; Molander et al., 1982).

Nerve transplantation experiments demonstrated that the neuronal environment must be conducive for regeneration (Aguayo et al., 1978; David and Aguayo, 1981; Kao et al., 1977; Richardson et al., 1982). Injured spinal cord and brain axons regenerate readily through grafted columns of Schwann cells in peripheral nerves but seldom enter grafted CNS nerve segments (Aguayo et al., 1978; Kao et al., 1977; Richardson et al., 1982). This indicates that in contrast to glial cells, the non-neuronal cells of the peripheral nerves (e.g., the Schwann cells) or components associated with them (e.g. diffusible or extracellular matrix) have the appropriate properties for regeneration. The contribution of these elements to regeneration is summarized.

2.1 Diffusible substances and nerve regeneration

Axonal injury causes changes in the activity of diffusible substances originating from the non-neuronal environment and also from the target organ. Thus, for example, the growth of the proximal stump of a transected PNS nerve is facilitated by diffusible proteinaceous molecules possibly anchored in the basal lamina and released from the distal stump of the transected nerve (Longo et al., 1983). Similarly, axonal injury induces increased neurite-promoting activity in extracts of the target organ (Giulian et al., 1986; Henderson et al., 1983; Nieto-Sampedro et al., 1984; Nurcombe et al., 1984). Furthermore, extracts prepared from denervated adult skeletal muscle contain an increased amount of neurotrophic activity which promotes survival of dissociated motor neurons and outgrowth of neurites from explants of spinal cord maintained in serum-free defined media (Nurcombe et al., 1984). This injury-induced increase in activity has also been observed in the brain. For example, the activity of a diffusible substance which is collected from the site of a brain

lesion and which affects the survival of chick sensory neurons in culture is increased after lesion (Nieto-Sampedro et al., 1983). Injury-induced increased activity is also manifested by the better survival of brain grafts in wound cavities several days after the injury (Nieto-Sampedro et al., 1983; Nieto-Sampedro et al., 1984).

Injury to optic nerves of fish (Rachailovich & Schwartz 1984) and peripheral nerves of mammals (Skene & Shooter, 1983) both regenerative systems, causes changes in type and amount of diffusible substances derived from surrounding non-neuronal cells. It is possible that among the various polypeptides which show variation in regeneration are components which are involved in glial activation for regeneration.

2.2 Extracellular matrix components and nerve regeneration

Extracellular matrices have been shown to have numerous functions in attachment and migration of cells during development and regeneration. Thus, the ability of axons to grow through tissue in vivo during development and regeneration may be regulated by the availability of extracellular matrix components. Among the various components laminin was found to have the most significant effect. It appeared that laminin expression is in correlation with growth (Hopkins et al., 1985; Liesi, 1985; Williams & Varon, 1985). Thus, while it is expressed continuously in adult fish optic nerve it is absence in mammalian adult optic nerves. It was therefore tempting to speculate that the absence of laminin in the CNS of mammals is a cause for their poor ability to regenerate which can be circumvented by external stimulus.

2.3 Injury in the fish optic nerve causes increased activity of substances which can trigger a regeneration like response in a non-regenerative system.

We have shown that soluble substances originating from regenerating fish optic nerve when applied to injured adult rabbit optic nerve cause in the later manifestation of early events of regeneration (Schwartz et al., 1985). The response includes changes in retinal protein synthesis and in growth activity in vitro. Moreover, similar effect is induced by media conditioned by newborn rabbit optic nerves (Hadani et al., 1984). We therefore proposed the existence of correlation between ability of nerves to grow and the ability of their environment to provide appropriate factors which were therefore termed by us as growth associated triggering factors (GATFs; Hadani et al., 1984). Based on those early results, the goal which we set for ourselves during the first year of this support was: (a) to determine the extent of the in situ morphological effect induced by the factors (i.e. by the conditioned media of the regenerating fish optic nerves or the newborn rabbit optic nerve) (b) to elucidate the possible biochemical changes induced in the nerves in addition to those already observed in the retinae (c) to find out the primary target of the applied substances and thereby to find out their mode of action (d) to get an insight to their possible biochemical nature.

The major findings towards any of these goals are summarized below in section 4.

3. Rationale for the approach taken in this work

In response to injury adult CNS exhibit several capabilities of both the glia and the axons which are needed for regeneration. Nevertheless these neurons rarely accomplish this process with functional recovery.

It is reasonable to believe that either all the needed events are occuring but not at the appropriate time and therefore lead to an abortive process or that one of the key elements is missing and therefore the other needed events don't occur.

Our strategy was to study a system which is endowed with a high regenerative capacity (i.e. the fish visual system) and thereby to get an insight to the mammalian CNS.

In the visual system of the fish we observed that injury causes environmental alterations. Based on these results we wanted to examine whether the regenerative supportive environment of a regenerating nerve i.e., goldfish optic nerve, can make a neuron of mammalian CNS (non-regenerative system) to express regeneration-associated response, having in mind that if successful, it would indicate that (a) a non-regenerative system is defective in its environmental factors, and (b) environmental changes in a regenerative system are involved in processes that enable production of factors needed for a regeneration. To carry out such a study, we developed a unique procedure of a transorbital surgery (Solomon et al., 1985) which enabled the use of an adult rabbit's visual system as our experimental model for a non-regenerative system.

At the onset of this study, we transplanted an optic nerve from a carp, which had been conditioned to regenerate by a crush injury, into a transected optic nerve of an adult rabbit by suturing the nerves end to end. However, immunological rejection was noticed after two weeks. To avoid the rejection, we used a "wrap around" with a silicone tube which was internally coated with collagen and contained diffusible substances (i.e. conditioned media) derived from regenerating optic nerves of fish (either carp or goldfish). As a result of this application the rabbit optic nerve and retina showed features which are characteristic of regeneration (Schwartz et al., 1985). These results raised the question as to whether injured optic nerves of adult rabbit have any potential to provide triggering factors. Our result showed that these nerves, do not

have the potential. Two possibilities therefore came to mind: either that intact nerves have this potential but lost it as a consequence of injury, or that intact nerves do not have the potential to provide the triggering molecules. In the latter case the potential could have been lost during maturation if produced during development. Our experimental approach of a synthetic "wrap around" implant provided the means to resolve this issue. Thus, we were able to show that media conditioned by newborn nerves have a similar activity, while media conditioned by intact or injured adult rabbit optic nerves showed minimal or no activity (Hadani et al., 1984).

It therefore seems that the ability of the axonal environment to provide triggering factors correlates with its growth state (regenerating fish optic nerve and neonatal rabbit optic nerve have the highest activity). This correlation justifies the consideration that inability of mammalian CNS to produce active factors may be a reason for their poor ability to regenerate.

Based on these observations it was essential to attack the following questions:

(a) What is the extent of the morphological response, induced in situ?

(b) Does the applied conditioned media have an effect on the environment of the injured nerves?

(c) What is the primary target of the applied factors?

(d) What is the identity of the factors?

4. Techniques

4.1 Surgical procedures

4.1.1. Carps:

Carps (Cyprimus Carpio, 800-1200g) purchased from Tnuva, Israel) were anesthetised with 0.05% tricaine methansulfonate (Sigma, Israel). Both optic nerves were then crushed with forceps, taking care to injure the nerve only and leave the surrounding tissue intact. Eight days after the injury, the injured nerves were dissected out and immediately transferred into serum free media (DMEM,GIBCO) for 1.5 h of incubation.

4.1.2 Rabbit:

(albino, The Weizmann Institute, breed Israel) were anesthesized by (5mg/Kg) xylazine and Ketamine (35mg/Kg) administered subcutaneously. The left optic nerve was then exposed using the transorbital surgical approach. The optic nerve was crushed (for 30 sec, with a hemostatic clamp) 4-6 mm distal to the eye globe, and a "wrap around" implant was immediately applied (Solomon et al., 1985). The "wrap around" implant consisted of a silicone tube (2mm and 4mm internal and external diameters, respectively; Burke, FRG), coated inside with collagen (Zyderm, Type V), which was soaked for 24h at 11°C in media containing the active diffusible substances (conditioning media) or the recovered fractions.

4.2 Preparation of conditioned media

The excised fish nerve segments were incubated for 1.5 h in serum free media. At the end of the incubation period the media (300 ul medium/nerves) were collected and stored at -20°C..

4.3 Analysis of Protein Synthesis in the Retina

All the rabbits, which were implanted either with the crude conditioned media or with the various recovered fractions, were sacrified one week after the surgery. The retinae on both sides were then separately excised and were processed for determination of total incorporation of [^{35}S]methionine or for profile of specific polypeptides by gel electro-

phoresis as was described previously (Hadani et al., 1984; Schwartz et al., 1985). The ratio L/R represent the specific activity of labeled protein in the retina of the treated (injured only or injured and implanted with conditioned media or recovered fractions) relative to the contralateral, non-injured, non-treated side. Each retinal preparation was then tested for protein profile by electrophoresis on acrylamide gel. For visualization of the labeled proteins the gels were treated with DMSO/PPO and fluorographed using Agfa Unit RP2 film.

4.4 Application of horseradish peroxidase

Rabbit optic nerves were crushed and wrapped around with silicone tubes containing media conditioned by optic nerves of neonatal rabbits (Solomon et al., 1985; Schwartz et al., 1985; Hadani et al., 1984). At various stages after this surgical manipulation horseradish peroxidase (HRP, type VI, Sigma) was applied to a new cut in the optic nerve, made proximal to the eye, between the optic disc and the primary crush injury. Application of HRP was accomplished by applying surgical sterilized oxidised cellulose (Ethicon) soaked in 30% HRP (in phosphate buffer, pH=7.4). Forty-eight hours after the HRP application, the rabbits were reanesthesized and perfused gravitationally with phosphate buffer (0.1M pH=7.4) through the exposed carotid artery. The whole retinas were then removed and mounted on precoated gelatin slides. The retinal slides were preincubated for 6 hrs in solution containing diaminobenzidine (DAB, 50 mg/100 ml), $CoCl_2$ (0.0025%), nickle ammonium sulphate (0.0002%) and dimethylsulfoxide (1%). At the end of the incubation the slides were transfered for 30 min into freshly-made phosphate buffer solution containing DAB (50 mg/100 ml) and covered with permount.

Animals were perfused further with 2% glutaraldehyde in phosphate buffer and the nerves were then excised from the optic disc to the chiasm. The excised nerves were incubated in phosphate buffer containing 30% sucrose. Cryosections (30 μm thickness) were taken and stained for HRP using diaminobenzidine as the chromophore of choice.

4.5 Thymidine incorporation into non-neuronal cells

Seven days after injury (which in some animals was followed by a wrap-around implant of GATFs) the animals were anesthetized and the nerves were dissected, including the intra-and extracranial parts. The nerves, from the globe to the chiasm, were cut into the following three segments: the proximal segment, from the globe to the crush site (about 4 mm); the medial, 4 mm distal to the site of injury, and the segment distal to it . For measurements of [^{3}H]thymidine incorporation, the segments were separately incubated for 2 h at 37°C in Dulbecco's Minimal Essential Medium (DMEM) containing [^{3}H]thymidine (10 uCi/segment; 40-80 Ci/mmol, Amersham). At the end of the incubation period the nerve segments were separately homogenized in a buffer containing Tris-HCl, pH 6.8, 5mM CDTA, 1mM DTT, and 1mM PMSF; all purchased from Sigma) in a glass-teflon homogenizer. The extent of incorporation was expressed by a ratio between the calculated incorporation (cpm/ug protein) into the proximal or medial segment and the distal segments.

4.6 Immunocytochemical analysis for laminin immunoreactive sites

Normal nerves and those that had been crushed seven days previously were removed and immediately placed into 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4, for 4 h at 4°C and then transferred into 30% sucrose for overnight incubation at 4°C. Eight micron cryostat sections were placed on gelatinized 8-spot slides (Shandon Scientific) and

treated as follows: Sections were exposed to PBS containing 1% BSA (for 3 h at room temperature or overnight at 4°C) and then with 0.15M Tris-HCl, 0.1M glycine, pH 7.2, (45 min at room temperature) to block non-specific binding of antibody and autofluroescence due to fixation. Serum of sheep containing antibodies against murine EHS sarcoma laminin (diluted 1:1000, gift of Dr. H. Kleinman, NIH) or PBS containing 0.2mg/ml BSA were then applied for 2 h at room temperature. Sections were washed (4x, 10 min each) in PBS containing 0.2 mg/ml BSA and then exposed to fluorescein isothiocyanate-conjugated rabbit $F(ab')_2$ anti-goat IgG (Cappel Laboratories, Penn., 20 ug/ml, also reacts with sheep IgG) diluted 1:100 for an additional 2 h at room temperature. The sections were mounted in Gelvatol 20-30 (Monsanto Corp., N.Y.) in tris-HCl, pH 8.2. Fluorescence was examined with a Zeiss Photomicroscope III using a 100 watt mercury lamp and Neofluar 25x objective. All fluorescent micrographs were taken using 1 min exposures on Kodak Tri-X film and were developed identically.

4.7 In vitro approach for laminin production

4.7.1 Laminin

Cells C-6 were grown in wells of microtiter plates in Dulbeco modified Eagles medium (DMEM) containing 10% fetal calf serum (FCS). When the cells reached confluency the medium was changed into Waymouth's medium supplemented with insulin (5 μg/ml), bovine serum albumin (BSA, free of fatty acids 0.5 mg/ml) and antibiotics (streptomycin/penicilin, 100μg/ml). The cells were kept at this medium for 24 h, followed by additional 24 h period in the same medium supplemented with media conditioned by the regenerating fish optic nerves. The cells were then examined for thymidine incorporation and laminin appearance.

4.7.2 ELISA screening methods for surface antigens

Cells, which were grown in microtiter plates, were centrifuged 3 min at 1000xg and then washed with phosphate buffered saline (PBS). Nonspecific sites were blocked by BSA for 30 min at room temperature. Following this incubation the supernatant was collected and antibodies at the appropriate dilutions were applied for 30 min. The cells were then washed with PBS and treated with the second antibodies conjugated to horseradish peroxidase (50 μl were added to each well for 1-3 h incubation at 37°C or overnight at 4°C). Cells were washed, and 100 μl of substrate was added to each well. Reaction was stopped by the addition of sodium azide (0.01 w/v) and citric acid (0.1M) Absorbance was recorded at 410 nm.

4.9. Steric exclusion chromatography

Conditioned medium of injured carp optic nerves was spun (1000xg, 2 min) and the supernatant was filtered through a 0.45 μm filter (Schleicher & Schuell, W. Germany). The filtrate was then applied to a Sephadex G-25 coarse column (2.5x50 cm equilibrated with 50 mM NaCl and 5 mM phosphate buffer, pH 7.4, Pharmacia, Sweden). Elution was performed with 50 mM NaCl and 5 mM phosphate buffer pH 7.4 at a flow rate of 96 ml/hr and fractions of 4 ml were collected. The eluate was monitored continuously by u.v. absorbtion at 280 nm.

4.10 Reversed-phase high performance liquid chromatography (RP-HPLC)

Fractions 81-120 from the Sephadex G-25 chromatography were pooled, lyophilized and redissolved in distilled water (2 ml). Acetic acid 0.12 ml was added, the solution was spun (10,000xg, 2 min) and the supernatant was applied to an octodecylsilane silica HPLC column (ultroshpere ODS, 5 μm 4.6 x 250 mm, equilibrated with 0.3% aqueous trifluoroacetic acid, Beckmann Instruments USA). THe column was washed with 10 ml of 0.3% tri-

fluoroacetic acid (Buffer A) at a flow rate of 0.5 ml/min and elution was then performed at 0.5 ml/min by a linear gradient of acetonitrile (0 to 10% during 60 min) followed by a gradient (10 to 50% during 10 min) in Buffer A. Fractions of 1 ml were collected. Protein was monitored by a fluorescamine monitoring system (Steins and Moschera, 1981) The amount of fluorescamine reactive material was determined in each fraction using glycine as a standard.

5. Summary of major results and achievements, and discussion

5.1. Morphological changes induced by GATFs

Morphological studies provided evidence that media conditioned by regenerating fish optic nerve (from now on will be termed GATFs defined above in 2.3) cause alterations in the injured adult rabbit optic nerve manifested by the prolonged survival of the injured fibers and by the appearance of growth cones (in contrast to their absence in the non-treated nerve) (Lavie et al., 1987).

Basically, the experimental paradigm used was the one which was described initially by us (Solomon et al., 1985; Schwartz et al., 1985; Hadani et al., 1984). Morphological analysis of the nerves was accomplished by electron microscopy and by light microscopy using horseradish peroxidase applied to the nerves at various stages after injury, into a new cut which was made proximal to the eye (see details in section 4.4).

Analysis of optic nerve sections revealed that, one week after the injury, labeled fibers were observed in both treated (Fig. 1b) and non-treated nerves (Fig. 1a). Two weeks after the injury, conspicuous differences were observed, manifested by the larger number of labeled fibers in nerves of experimental animals (Fig. 1 d vs c). One month after injury, a few axonal fibers were seen on the periphery of the experimen-

tal nerves, (Fig. 1f,g,h) while in the control nerves, no labeling could be detected (Fig. 1e). These results were further substantiated by electron microscopic studies which revealed fibers with typical growth cones embedded in glial cells. It therefore appears that the glial cells, as a result of being exposed to the CM, acquired a surface supportive for growth (This issue is discussed under section 5.2.2).

The results imply that one month after injury, the observed labeled fibers reflect growth caused by the GATF-implantation, rather than survival. This would suggest that the active component(s) in the conditioned medium may affect both survival of fibers after injury as well as their growth. It is not known whether this growth is directly caused by GATFs or is secondary to GATF-induced neuronal survival. Furthermore, independently of whether the primary effect of GATF is survival or growth, it is possible that it is mediated via a cascade of events which is triggered by GATFs. One such cascade of events may involve the direct activation of glial cells surrounding the injured nerve, thereby creating growth supportive glial cells or reactive glial cells. Our preliminary results which support this notion are summarized in 5.2.2 and 5.2.3).

5.2 GATFs induces environmental changes associated with growth

In an attempt to find out whether media conditioned by regenerating nerves have the capacity to activate glial cells, we analyzed the neural environment subsequent to the application of GATFs. Environmental changes were examined by measuring [^{3}H]Thymidine incorporation (indicative of cell division) (Pellegrino & Spencer, 1985; Spencer et al, 1981) by electrophoretic analysis of the nerve components, and by immunocytochemically looking for extracellular matrix components.

5.2.1 GATFs and thymidine incorporation

It appeared that injury of adult rabbit optic nerves resulted in an increased incorporation of thymidine, manifested primarily in the proximal segment adjacent to the globe. Such an increase could be the result of any of the following events or combination thereof: proliferation of glial cells, invasion of connective tissue-derived cells, and an inflammatory response.

In order to examine the effect of GATFs on an incorporation, we compared the effects of GATF-containing and GATF-free silicone tubes (designated in Table 1 by crushed+CM and crushed+M, respectively) on the proliferation. GATF-free tubes caused a significant decrease in the index of proliferation (Table 1). This decrease may be the result of a physical barrier, created by the silicone tube, which interferred with the migration and invasion of cells.* In contrast, GATF-containing tubes caused increased proliferation up to values which were not significantly different from those in the injured non-treated nerves (Table 1). The observation that the silicone tube served as an insulator, taken together with the fact that the only cells within this insulator were the perineural cells, indicate that the latter are the cells which underwent changes in thymidine incorporation induced by GATF.

5.2.2 GATFs induce changes in the nerve protein composition.

Analysis of protein profile of the various treated nerves was carried out by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). GATFs caused a striking change in the nerve protein profile. Most prominent was the appearance of an abundant polypeptide of 12 kDa primarily in the proximal nerve segment (Fig. 2, lanes 4,5 vs. 2 3). This polypeptide,

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* In aggreement, empty silicone tubes were recently used as a means to impede scar formation in injured peripheral nerves using polyglactin tube (Billingsley and Mandel, 1982)

the major band in lane 5, could not be detected in optic nerves of intact adult rabbit (lane 1) and it was detected to only a marginal level after injury and application of a silicone tube free of GATFs (lane 3; Bawnik et al.,, 1987).

The 12 kDa polypeptide may be axonal-derived. Alternatively, it may be a released or a cytoplasmic protein derived from the non-neuronal cells. As such the 12 kDa polypeptide may serve as a second message in the cascade of events initiated by GATF and leading to the expression of regeneration like characteristics.

The resolution of biochemical methods which have been used to monitor the environmental changes caused by GATFs have not allowed detection of possible quantitative differences in composition of the extracellular matrix. They also offer no information about possilbe changes in distribution of any one specific extracellular component.

Extracellular matrix components were shown to play an important task in nerve growth both in vivo and in vitro. To investigate possible GATF-induced alterations in the extracellular matrix, we decided to employ immunocytochemical tools. Specifically, we examined the presence of laminin immunoreactive sites since laminin is a matrix component which, in a regenerative system, contributes to the formation of a growth supportive milieu (Williams and Varon, 1985; Manthorpe et al., 1983; Hopkins et al., 1985; Davis et al., 1985; Lauder et al., 1986; Leisi, 1985).

Towards this end, we carried out immunocytochemical studies aimed at finding out whether there is any active component in GATFs which can directly activate glial cells to synthesize or to synthesize and accumulate laminin.

5.2.3 Laminin immunoreactive sites are induced by GATFs

Adult rabbit optic nerves were removed and examined immunocytochemically using goat antibodies directed against mouse laminin. Intact nerves and two kinds of injured optic nerves were studied. The latter include nerves that had been crushed and then implanted with a silicone tube containing collagen soaked in fresh medium and nerves that, subsequent to crush, were implanted with a silicone tube containing collagen saturated with GATFs (Solomon et al., 1985). Medium conditioned by regenerating fish optic nerves served as the source of GATF. This preparation was shown to be free of laminin (unpublished observations).

Our results reveal that, in cryostat sections of intact adult rabbit optic nerve, laminin immunoreactive sites appear in the epineurium (Fig. 3d, arrow) and around the sparsely distributed blood vessels (not shown). Following injury, laminin immunoreactivity could be detected in the epineurium, in connective tissue surrounding what appeared to be an increased number of blood vessels and in coarse bundles of scar tissue (Fig. 3b., arrow). However, after injury and application of GATF, the pattern of laminin immunoreactivity was changed and additional laminin reactive sites could be detected. These appeared as a fine network coursing throughout the matrix of the nerve at the actual site of injury as well as sites proximal, but not distal, to it (Compare Figs. 3a and 3c).

It thus appeared from this study that the environmental changes caused by GATFs (derived from regenerating fish optic nerve) include those which lead to accumulation of laminin, an extracellular matrix component known to play a role in support of neural growth and elongation (Lander et al., 1985; Williams and Varon, 1985). Without application of

GATFs laminin immunoreactive sites are not present within the matrix of the injured adult rabbit optic nerve. The more widespread distribution of laminin may be directly induced by the GATFs or may result from a cascade of events initiated by the GATFs. It could involve increased production or secretion of laminin or other metabolic changes which affect its accumulation.

Our preliminary results, as well as reports in the literature, reveal that, in intact fish optic nerve (a regenerative system), in contrast to rabbit, laminin immunoreactive sites are present throughout the nerve matrix mainly in tongues of connective tissue which project in, from the periphery (Liesi, 1985; Hopkins et al., 1985). The continuous presence of laminin in the fish, like other components needed for growth, may be associated with the general plasticity in this system (Bawnik et al., 1986; Quitschke and Schechter, 1984). It therefore appears that the absence of laminin in the matrix of the injured mammalian optic nerve may be a cause, partially circumventable by GATFs, for its poor inherent regenerative ability. Further studies are needed to identify the laminin immunoreactive sites in fish and rabbit, and to elucidate the mechanisms which regulate its accumulation in the adult fish optic nerve (a regenerative system) and absence from the adult rabbit optic nerve (a non-regenerative system).

5.4 GATFs can activate directly the non-neuronal cells of the injured nerve.

The increase laminin immunoreactive sites seen in GATF-treated injured nerves suggested that substances derived from regenerating fish optic nerves contain stimuli that can activate astrocytes of adult mammals to produce or to produce and to accumulate laminin. To test this

possibility we have established in the last three months conditions of glial cells cultures.

These culture conditions allowed us to examine directly whether there are components within the CM of the regeneating fish optic nerve which can activate glial cell and thereby cause their transformation towards cells supportive for growth, at least with respect to laminin. Our preliminary results are in favor of this possibility. These studies were carried out so far using glioma cell line (C-6) and primary brain astrocytes. The results shown in Fig. 4 summarize our preliminary results which suggest that CM of regenerating fish optic nerve stimulates these glioma cells to produce laminin. Further studies are currently being carried out to find out whether the substances responsible for this activity are copurified with the substances involved in the observed early biochemical response induced in situ and, if not, what is their possible relationship.

5.5 Isolation of a molecule which is active in situ

Isolation of an active component from media conditioned by regenerating fish optic was accomplished by two steps which included gel filtration and high performance liquid chromatography (HPLC).

Preliminary results with various types of gel filtration chromatography revealed the existence of several active molecular species within this preparation, one of which is a low molecular weight component. This low molecular weight component was obtained after molecular sieving chromatography on Sephadex G-25 and was further chromatographed on reverse phase-HPLC in which acetonitrile was used as the solvent for separation.

Scale up purification resulted in one peak of activity which retained only part of the activities of the original conditioned media

(Fig. 5). This low molecular height substance (about 300 daltons) was found to be fluorescanime positive and to have an intrinsic fluorescence.

Further studies are in progress using spectroscopic methods and amino acid analysis will be used to identify this molecule. The bottle-neck is having the factor in quantities. Moreover, other activities within the conditioned medai are also in process of purification.

6. Conclusions

Two years ago we showed that substances originating from a regenerative system can trigger a regenerative response when applied to a non-regenerative system.

During the first year of this support from the U.S. Army we were able to reach the following:

(i) the regenerative response induced by the soluble factors is manifested *in situ* by the appearance of new growth (e.g. growth cones) in the treated nerves, in an area which otherwise appeared to be comprised of scar tissue. It was therefore suggestive that our surgical manipulation contributed to the alteration of the glial cell surface and thereby made the glial cells supportive for growth (Lavie *et al.*, 1987).

(ii) Based on the results summarized in (i) efforts were made to analyze the nature of the alterations which the glial cells underwent. It appeared that the applied soluble substances caused an increase in thymidine incorporation, and appearance of changes in the nerve-derived proteins (Bawnik *et al.*, 1987).

(iii) The applied soluble substances caused an increase in laminin distribution in the injured nerve (Zak *et al.*, 1987). Laminin is a protein which is important in growth and elongation of nerve fibers both *in vitro* and *in vivo*. The increase laminin may be a critical step circumvented by

the active implanted factors. The quiescent glial cells are activated by soluble factors.

(iv) We further investigated the possibility that the production of laminin is directly activated by the applied soluble substances. This issue has begun to be examined in vitro using glial cell cultures.

(v) Our preliminary results suggest that in the media conditioned by regenerating fish optic nerves there are several factors working either consecutively or in concert. We have already isolated one such factor and purified it to homogeneity but we have not identified it yet. Furthermore, we have already made the first steps towards purification of the factor which activate directly the glial cells.

Our further goal is to isolate and identify the various active substances both in vitro and in vivo and to find their mode of action, their possible mutual relationship, and to devise an optimal way for their application in order to enhance growth and elongation.

LEGEND TO FIGURES.

Figure 1: HRP-labeled optic nerves.

Longitudinal cryosections were taken through the optic nerve and labeled fibers were visualized with HRP using the Hanker Yates method (ref).
a,c,e -represent nerve sections taken one week, two weeks and one month, respectively, after the optic nerve crush.
b,d,f,g,h-- represent labeled nerves taken at the same time intervals as above, but after injury and implantation of silicone tube containing GATFs. Note the paucity of labeled fibers in the implanted injured nerve (f-h) and their absence in the control (e). The arrows are pointing to structures which resemble growing fibers.

Figure 2: GATFs induce the appearance of a 12 kDa polypeptide.

Nerve homogenates (16 ug protein) of the various experimental groups were applied to SDS-polyacrylamide (15% acrylamide) gels. After electrophoresis, the gels were fixed and stained. The intensity of labeling of 12 kDa polypeptide, as determined by densitometric scanning of the gel at 560 nm (Gilford Model?), was ca. 30 times higher in the proximal segment of the GATF-treated nerve relative to the GATF-nontreated nerve. In 9 of 13 GATF-treated rabbits, the 12 kDa polypeptide appeared as a prominent protein band. In none of control rabbits (about 20 tested ones) could such an increase be detected.

Figure 3: Immunocytochemical staining using anti-laminin antibodies.
a) Section taken from area proximal to site of crush and subsequent application of GATFs. Arrows point to blood vessels delineated by the

anti-LN antibody; asterisk marks area where a fluorescent network can be seen in the injured nerve matrix. b) Section from area proximal to site of crush of nerve treated with wrap around silicone containing collagen soaked in control medium. Arrow marks a blood vessel in scar tissue. Note absence of immunofluorescence in nerve matrix (asterisk). c) Section from area distal to site of crush and application of GATF. d) Section from normal contralateral nerve. Arrow points to immunoreactivity in the epineurium. e,f) Phase and fluorescence micrographs of the same section treated as above except for application of specific primary antibody. scale bar = 400um.

Figure 4 Laminin immunoreactivity in C-6 cells treated with media conditioned by regernerating optic nerve.

Cells were grown in microtiter plates. Twenty four hours after treatment either with conditioned media or with FCS they were washed and incubated with rabbit anti-laminin antibodies or with preimmune mouse sera. Determination of bound antibodies was performed by HRP-conjugated goat anti rabbit antibodies and the chromophone. The results were recorded spectrophotemetrically at 430 nm. Each point represent an average + SD from three wells. This experiment was repeated three times. A, represent s the binding to laminin specific antibodies and to pneimmune sera of cells kept in the basic growth medium only. B, representsthe binding to cells stimulated with 10% FCS; C, cells stimulated with 0.1μg/ml of conditioned media; and D, cells stimulated with 1μg/ml of conditioned media.

Figure 5. Reversed-phase HPLC. Combined and lyophilized fractions 81-180 from the steric exclusion chromatography (Fig. 1B) were dissolved

in aq acetic acid (1M 2 ml) and applied to an octodecyl silica HPLC column (ultrosphere ODS 5 μm 4.6x250 mm Beckmann Instruments). The column was preequilibrated in 0.3% aq trifluoroacetic acid and elution was done at a flow rate of 0.5 ml/min by a linear gradient of acetonitrite (0% to 10% during 60 min) followed by a step of 10% to 50% during 10 min. Fractions of 1 ml were collected. The effluent was monitored by post column reaction with fluorescamine which consumed 1-2% of the column effluent. Biological activity was found in fractions 33 and 34.

Table 1: Thymidine Proliferation Index in Response to GATFs Application

	Intact	Crushed	Crushed + M	Crushed + CM
Proliferation index	a 1.02±0.07	b 1.80±0.10	c 1.20±0.08	d 1.66±0.09
Number of animals	4	5	6	9

a and b are significantly different with a p value < 0.01; c and d are significantly different with p value < 0.05.

Rabbit optic nerves were crush injured unilaterally 4-5 mm distal from the eye globe. Some of the nerves remained untreated, others were "wrapped around" with silicone tubes containing either collagen and media (crushed + M) or collagen and media conditioned by regenerating fish optic nerves (crushed + CM). One week after the surgery, the nerves were excised and cut into three segments. Each segment was separately incubated in media containing [^{3}H]thymidine. Proliferation index is expressed by the ration between cpm of ^{3}H-Thymidine incorporation/mg protein in the proximal segment and in the distal segment.

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Figure 1: HRP - Labeled Optic Nerves

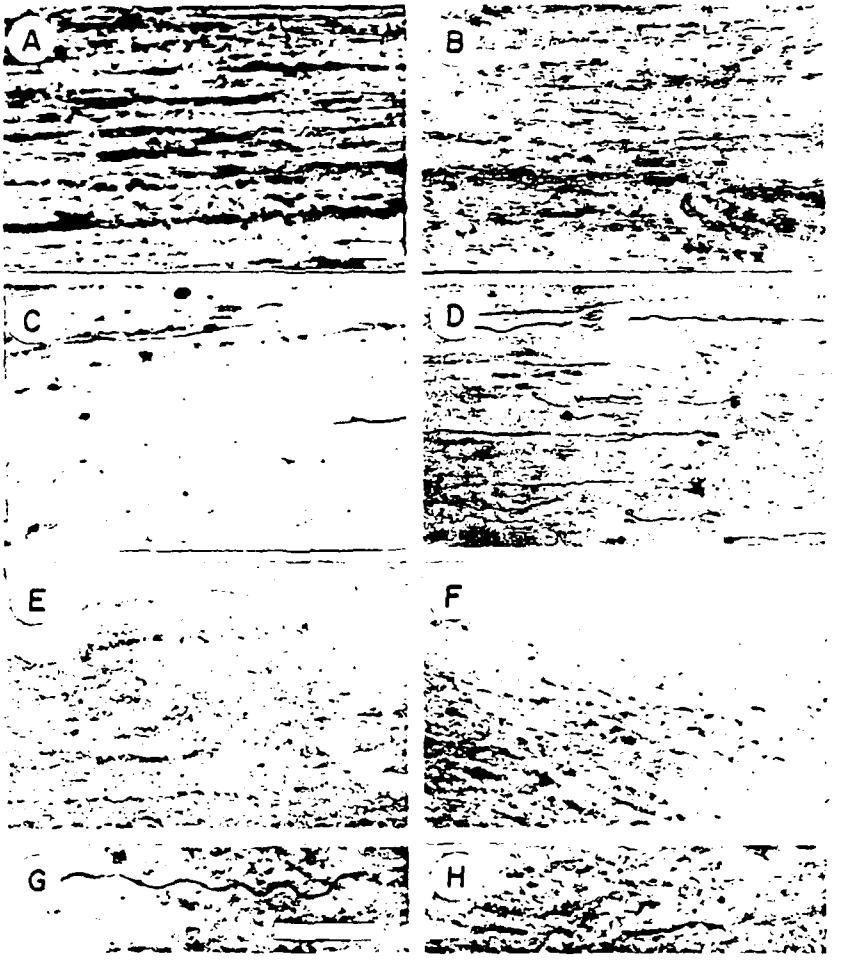


Figure 2: GATFs Induce the Appearance of a 12kDa Polypeptide

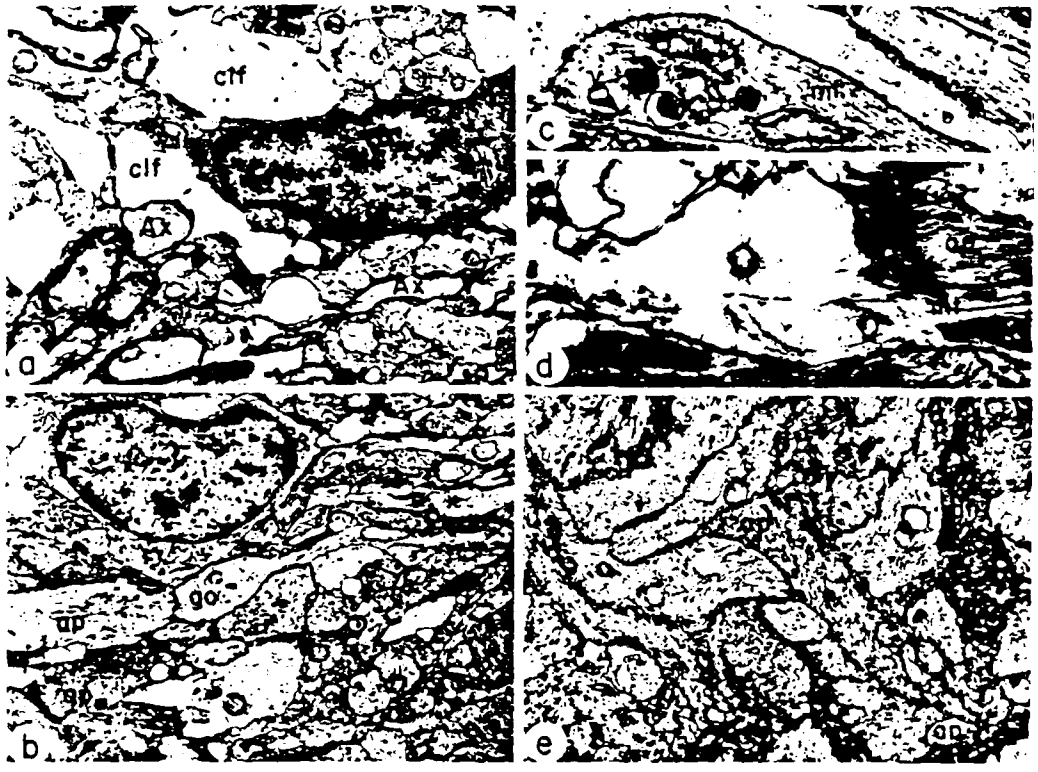


Figure 3: Immunocytochemical Staining Using Anti-Laminin Antibodies

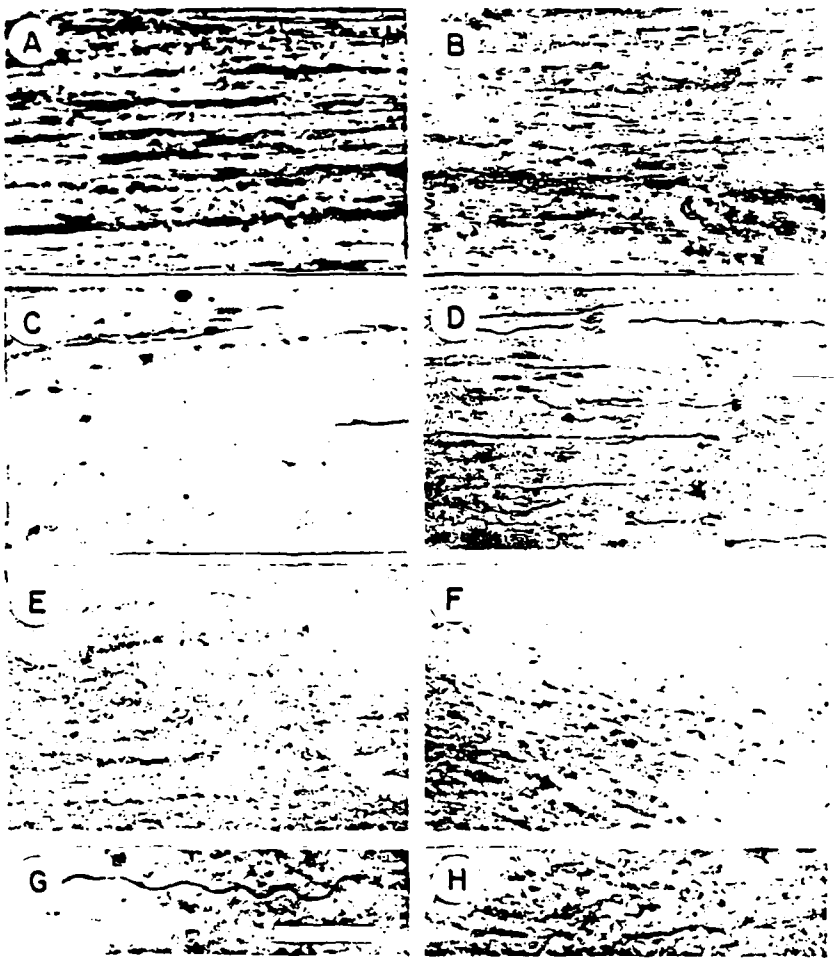


Figure 4: Laminin Immunoreactivity in C-6 Cells Treated With Media Conditioned by Regenerating Optic Nerve

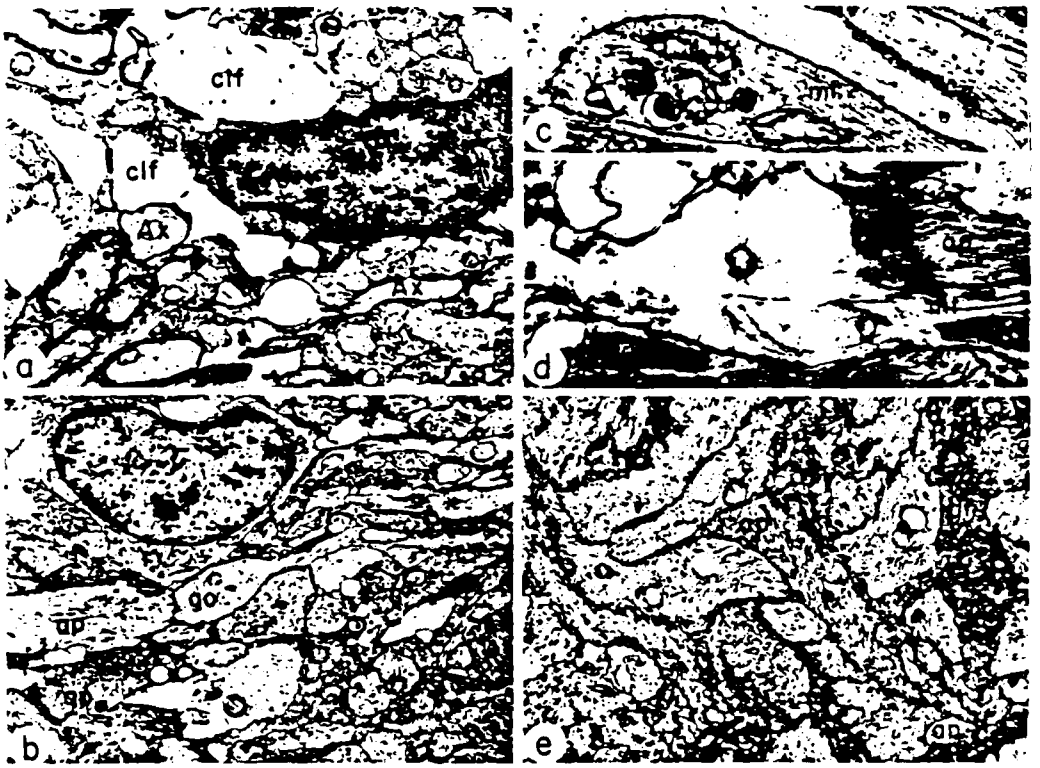


Figure 5: Reverse-phase HPLC

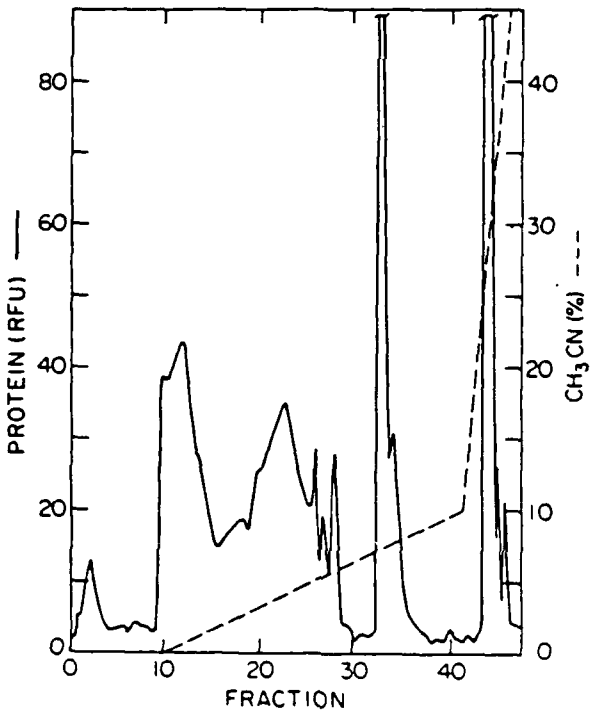
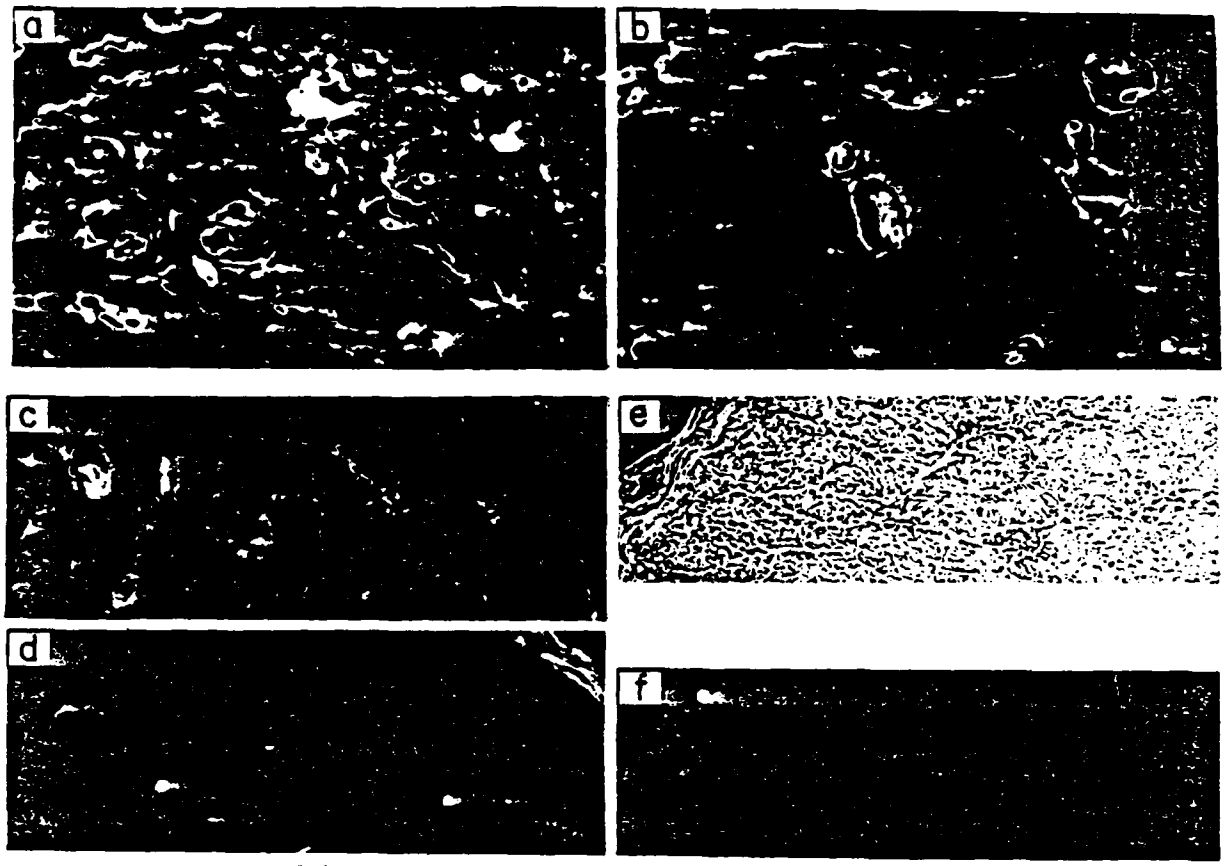


Figure 6: Reversed-Phase HPLC



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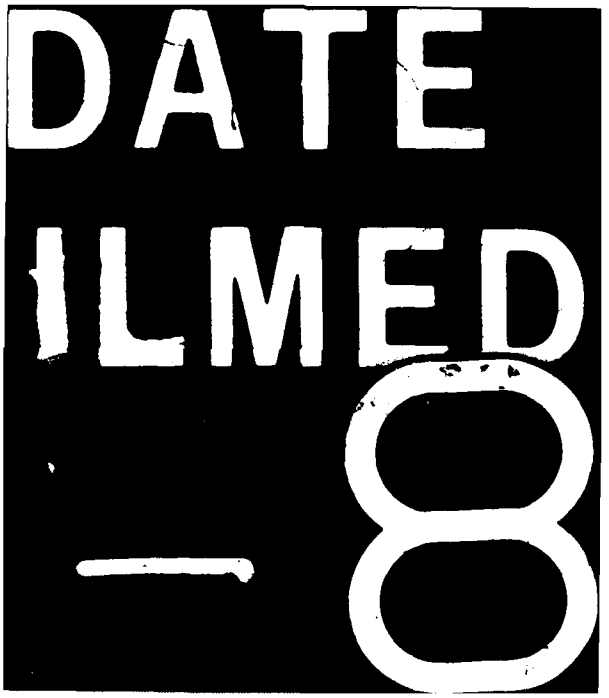
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